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Analyses of Conditions for KMSSS Loop in Tyrosyl-tRNA Synthetase by Building a Mutant Library

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The KMSKS motif is the ATP binding motif for aminoacylation process of class I aminoacyl-tRNA synthetases. Although researches based on natural proteins inform us about the contribution of natural amino acid sequences for the catalysis, they have difficulties in discussing the other alternative sequences and prohibited sequences for the motif to maintain the catalytic ability. In order to reveal such the conditions for the alternative and prohibited sequences, it is important to investigate a library of various mutants for the motif. For that purpose, we build a library of more than 200 mutants substituting the KMSSS loop, Lys204-Met205-Ser206-Ser207-Ser208, in tyrosyl-tRNA synthetase of *Methanococcus jannaschii*, and their catalytic abilities were examined by the Amber suppression method. Mutants of K204R and K204N still maintained catalytic abilities to a certain extent. On the other hand, a variety of alternative sequences for Ser206-Ser207-Ser208 were obtained, and some of those did not include either Ser or Thr, which were regarded as necessary residues in the KMSKS motif in previous works. In this article, catalytic activity of all the mutants are represented in detail and some suggestions for the condition of the motif are discussed.

Key words: amino-acyl tRNA Synthetase, ATP binding motif, KMSKS loop, amber suppression, mutational analysis.

Today's bioinformatics approaches in protein science generally employ statistical analyses of the databases based on sequences and structures of natural proteins. Those approaches have been proved to be quite successful in finding motifs and other conserved sequences which are important for the catalytic activation of proteins. Although most of the genome information from such the databases has been acquired from natural proteins, it would be possible to say that such amino acid sequences of the motifs obtained can be one of the local optimal sequences. In other words, it may be possible that sequences which differ from the wild-type motif would have catalytic activities at the similar degree as the wild-type motif. In order to reveal how the motif works in the proteins, it is important to investigate the common feature among those mutant and the wild type motifs. It is also important to investigate the difference in the sequences between catalytically active and inactive mutant motifs. For that purpose, it would be quite significant to build a library of mutant proteins and to analyse such data by bioinformatics approaches.

KMSKS motif is a signature motif involved in ATP binding interactions, and the amino acid sequences of the loop are conserved among class I aminoacyl-tRNA synthetase (aaRS) such as TyrRS, CysRS, MetRS, TrpRS (*1–4*) (Table 1). The motif is also conserved among different species of eubacteria, archaebacteria and eukaryote (*1–6*). In particular for bacteria, the second lysine in the KMSKS motif is highly conserved among the class I aaRSs (*6*). Hountondji *et al*. (*5*) suggested a hypothesis that SKS sequence in the KMSKS loop of class I aaRS corresponds to the GKT/S sequence of the ATP binding sites of many proteins. Those results were good examples of the motif search analyses, and other numerous previous researches had proved effectiveness of the motif search analyses.

So far, a lot of researches on KMSKS motif in class I aaRSs were performed on theoretical (*7–14*) and structural (*15–24*) bases. Among them, bacterial TyrRSs have been studied extensively, and catalytically important residues and their structural formations have been identified. The second lysine in the KMSKS motif is highly conserved among the class I aaRSs (*6*) and interacts with the α-phosphate of the ATP in the transition state (*15*). The mutation of second lysine residue to alanine destabilizes the transition state for tyrosine activation by 3.0 kcal/mol (*7*, *9*). The mutation of the first lysine to alanine, and the mutations of successive serine or threonine to alanine in the KMSKS motif revealed the importance of the first lysine and successive serine or threonine in the motif (*11*). Structural analyses revealed that induced-fit conformational rearrangement of the flexible KMSKS loop occurs during the catalysis of Tyr-AMP formation (*7*, *17*).

The most of eukaryotic and some of archaeal TyrRS lack the second lysine of the KMSKS motif such as KMSSS in human TyrRS. As the difference between KMSKS and KMSSS, Austin and First revealed that the KMSSS loop requires potassium for the catalysis, despite the fact that their overall catalytic efficiencies are similar. Mutations of the three serine residues of the KMSSS loop also revealed that the side chains of

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the first and the third serine residues play important roles in the Tyr-AMP formation (*14*).

Those works revealed some important features of the KMSKS motif. By adding the knowledge obtained from mutant library to the knowledge from the wild-type library, more interesting idea will be suggested. In this article, it is assumed that the loop can be mutated without deforming the frame structure of the aminoacyl-tRNA synthetase.

EXPERIMENTAL PROCEDURES

Building a Mutant Library by Randomized PCR—Sequences of KMSKS loop is Lys204-Met205-Ser206-Ser207-Ser208 in *Methanococcus jannaschii*, Lys-Met-Ala-Ser-Ser in *Methanosarcina mazei* and Lys-Met-Ser-Ser-Ser in *Human*. In this article, we would like to investigate the condition for KMSKS loop by focusing on the mutants derived from TyrRS associated with *Methanococcus jannaschii* (MjYRS). A library of more than 200 mutants was built by mutating nine residues from Glu202 to Gly210 in MjYRS which covers KMSKS corresponding residues of Lys204 to Ser208 (Tables 3–6). At first, residues were grouped by three residues as Glu202-Gly203-Lys204, Lys204-Met205-Ser206, Ser206-Ser207-Ser208, and Ser208-Lys209-Gly210, and the three residues of each group were randomly mutated (Tables 3 and 5). Besides them, a group of five residues as Lys204-Met205-Ser206-Ser207-Ser208 were also mutated randomly (Table 5).

Table 1. **Variations of KMSSS loop from alignment of the KMSKS regions.**

TyrRS (*Methanococcus jannaschii*) Amino acids	Expressions in other species
K	(25)K, (5)V, (2)ER, (1)ADIMP, one gap
M	(17)M, (11)L, (5)F, (2)Q, (1)DEKIR
S	(26)S, (14)G
S	(39)K, (1)T
S	(19)S, (14)T, (6)R, (1)N
K	(11)K, (7)L, (6)R, (4)E, (2)DPVY, (1)AHIT
G	(14)G, (4)SV, (3)AH, (2)ELNY, (1)DKIT

Alignment data is from ref. (*5*), which consists of archaeal (1 entry), bacteria (27 entries) and eukaryote (12 entries). In the table above, '(39)K' indicates that Lysine was found from fourteen species where *M. jannaschii* was Ser.

In synthesizing the whole gene of MjYRS mutants, the gene was divided into two parts. The former part of the gene consists of gene that preceding the mutation sequence, and the later parts of the gene consists of remaining part including mutation sequence and overlapping sequence to the former part. Thus, the former part is common among the all mutants. Synthesis of whole gene is performed by overlap PCR method (*25–27*). At first, the former part is synthesized by using Mj-BF and Mj-BR primers (Table 2), and the part is commonly used for all the mutants. The later part is synthesized by using Mj-EF and Mj-ER primers. EF primers have a variation as Mj-EF-xxXMSSS, Mj-EF-XXXSS, Mj-EF-KMXXX, Mj-EF-KMSSXxx, Mj-EF-XXXXX corresponding to randomizing categories, but Mj-ER primer is commonly used for all the mutants (Table 2). In synthesizing the former and later parts individually, wild-type gene of MjYRS was used for PCR template. Whole the gene of the mutant is then synthesized by overlap PCR method using Mj-BF and Mj-ER as primers and the former part and later part of the gene as templates.

Mj-BF and Mj-EB primers have cloning site sequences for restriction enzymes NdeI and BglII respectively. Genes of MjYRS mutants and tRNA for the Amber suppression (MjR1) (*28*, *29*) were inserted into a vector plasmid derived from pACYC184.

Colony Selection by the Amber Suppression Method—Activities of the mutants were examined by the Amber suppression method (*28*, *29*). The gene of the chloramphenicol resistant protein (CAT) was coded in a vector plasmid derived from pBR322. The gene of CAT is inserted into this vector, and codon of the 10th amino acid in CAT gene is mutated to Amber codon (*28*, *29*). Amber suppressor tRNA (MjR1) is so designed that it can recognize amber codon, and it can be recognized by wild-type MjYRS. MjR1 is also designed not to be recognized by animoacyle-tRNA synthetases coming from the *Escherichia coli* competent cell.

The vector with CAT mutant gene is transformed into *E. coli* competent cells accompanied by the vector with MjYRS genes. If a mutant of MjYRS has an activity to aminoacylate MjR1 with tyrosine at the position of Amber codon, the chloramphenicol-resistant protein will be expressed successfully, and the competent cell can

Table 2. **Residual groups of random mutations and PCR primers to designed mutant library.**

Residual groups of mutation	Primers	Primer sequences
Glu202-Gly203-Lys204	Mj-EF-xxXMSSS	GTCTTAACGGGTTTGGATGGAnnknnknnk ATGAGTTCTTCAAAAGGGAATTTTATAGCTG
Lys204-Met205-Ser206	Mj-EF-XXXSS	GTCTTAACGGGTTTGGATGGAGAAGGAnnknnknnk TCTTCAAAAGGGAATTTTATAGCTG
Ser206-Ser207-Ser208	Mj-EF-KMXXX	GTCTTAACGGGTTTGGATGGAGAAGGAAAGATG nnknnknnkAAAGGGAATTTTATAGCTG
Ser208-Lys209-Gly210	Mj-EF-KMSSXxx	GTCTTAACGGGTTTGGATGGAGAAGGAAAGATGAGTTCT nnknnknnkAATTTTATAGCTGTTGATGAC
Lys204-Met205-Ser206-Ser207-Ser208	Mj-EF-XXXXX	GTCTTAACGGGTTTGGATGGAGAAGG AnnknnknnknnknnkAAAGGGAATTTTATAGCTG
Common primers for all the mutants	Mj-BF	CCAGCCTGATGTAATTTATC
	Mj-BR	TCCATCCAAACCCGTTAAGAC
	Mj-ER	AGGGTCAATGCCAGCGCTTC

survive on the culture plate rich in chloramphenicol. If a mutant of MjYRS does not keep catalytic ability, its transformed *E. coli* cell cannot survive on the plate. In this experiment, chloramphenicol of 300 μg/ml was dissolved in LB culture plate.

Colonies selected from chloramphenicol rich culture plates will be sequenced and determined the amino acid sequences which maintain the catalytic activities of TyrRS (Tables 3–6).

Adding Specifically Designed Mutants to the Library—Amino acid sequences of the above selected colonies tell what kinds of sequence can have the ability of TyrRS. However, in order to determine what is necessary and what is prohibited for the loop, it is important to investigate sequences of the loop having poor catalytic abilities. For that purpose, we designed more than two hundred mutants individually to add to the library (Table 7). For example, mutant samples 1–19 in Table 7 are designed to mutate the first Lys to the other kinds of amino acids in order to investigate the importance of the first Lys.

Some of those specifically designed mutants should maintain catalytic abilities of the loop, and the others should have poor catalytic abilities. By analysing the difference in amino acid sequences between mutants with rich and poor catalytic abilities, it becomes possible to determine those boundary conditions.

Table 3. **Expressions of selected colonies from the Amber suppression screening: Randomized mutations around the KMSSS loop.**

xx**X**MSSS		**KMSS**Xxx		X**MSSS**X
AWK	QAK	SKG	MLL	K____K
CGK	RIK	ALN	MSL	K____A
EGK	RRK	ARV	RAN	K____E
FTK	STK	AWA	RGD	K____F
GLK	TGK	CGS	RLG	K____G
GVK	TVK	GGG	RLG	K____H
GWK	VIK	GGY	RWG	K____K
HAK	VVK	GHR	SAA	K____L
IAK	WGK	GLA	SFR	K____R
LAK	YCK	GLV	SNR	K____S
LYK	YGK	GRC	SRN	K____T
MKK	YNK	GSV	SRY	K____V
PTK	YRK	HRL	SYC	
		IVG	TNK	
		KGW	VAS	
		LGL	VGW	
		LRG	YSG	

Table 4. **Numbers of expressions at each residual position around the KMSSS loop.**

Residual numbers	Wild-type amino acids	Expressions from survived colonies
202	E	(4)Y, (3)G, (2)LRTV, (1)ACEFHIMPQSW
203	G	(5)G, (4)A, (3)TV, (2)WIR, (1)CKNWY
204	K	(38)K
205	M	
206	S	
207	S	
208	S	(8)GS, (7)R, (4)A, (3)KLV, (2)HMT, (1)CEFIY
209	K	(8)G, (6)LR, (3)A, (2)SNW, (1)KFHVY
210	G	(7)G, (4)L, (3)ANRV, (2)CSWY, (1)DK

In the above table, '(20)G' in the row labeled as 206 indicates that Gly was found from twenty clones at residual number 203 where the wild type residue was Ser.

Table 5. **Expressions of selected colonies from the Amber suppression screening: randomized mutations of the KMSSS loop.**

xxxxx		xxx**SS**		**K**xxx**S**		**KM**xxx			
KMSSS	4	KMS	4	MSS	4	SSS	4	GSS	3A
KCSSS	3B	KAA	4	ASC	3B	AAS	4	GTA	4
KMSST	4	KAG	3A	ASS	3A	ACS	3B	GTF	3A
KQSAS	3B	KAS	3A	AST	3B	ASF	3B	GTS	3A
PLGRS	0	KCA	3A	MAT	3B	ASG	4	GVC	3B
		KCS	3B	MGN	3B	ASM	4	*GVL*	2
		KMA	4	MSC	3B	ASR	3B	*GVP*	1
		KMC	4	MST	4	AST	3B	GVS	3B
		KMG	4	QSS	4	ATS	3B	SAG	3B
		KMS	4	QST	3B	CSS	4	SA S	3B
		KMT	3B			GAC	3A	SCS	3B
		KQA	3B			GAS	4	SCT	3B
		KSA	3B			GAT	3B	SSC	3B
		KSS	3A			GCA	3A	SSF	3B
		RMG	2			GCS	3B	SSG	4
		SMS	1			GKS	3B	SSQ	4
						GNS	3B	SSV	4
						GSA	3B	STA	3B
						GSC	3A	STC	3B
						GSF	3A	ST S	4
						GSG	3B	STW	3B
						GSH	4	STY	3B
						GSQ	3B		

Left column: Sequences of mutants, Right column: degrees of activities by amber suppression (in order of increasing activity: 0, 1, 2, 3B, 3A, 4).

Table 6. **Numbers of Expressions at each residual position of the KMSSS loop.**

Residual numbers	Wild-type amino acids	Expressions from survived colonies
204	K	(18)K
205	M	(13)M, (6)A, (4)Q, (3)C, (1)S
206	S	(32)S, (22)G, (14)A, (2)C, (1)T
207	S	(25)S, (13)T, (7)AC (2)NV (1)K
208	S	(18)S, (5)C, (4)AGFT, (1)HMQRVWY

Evaluation of Catalytic Abilities by Spotting Test of the Amber Suppression—In order to examine degree of catalytic activity of each MjYRS mutant properly, we employed spotting test of the Amber suppression method (Supplementary Material). In this test, plasmids inserted a single mutant for MjYRS are transformed into *E. coli* competent cells, and the cells are cultured on the chloramphenicol free plate overnight. Three colonies are picked up from the plate, and they are mixed into

Table 7. **Activity degrees of ATP interaction of mutants.**

1.	AMSSSK	1	50.	KNSSSK	1	98.	KCGVLK	1
2.	CMSSSK	1	51.	KPSSSK	0	99.	KDGVLK	0
3.	DMSSSK	1	52.	KQSSSK	4	100.	KEGVLK	0
4.	EMSSSK	1	53.	KRSSSK	0	101.	KFGVLK	0
5.	FMSSSK	0	54.	KSSSSK	3B	102.	KGGVLK	0
6.	GMSSSK	1	55.	KTSSSK	2	103.	KHGVLK	0
7.	HMSSSK	1	56.	KVSSSK	1	104.	KIGVLK	0
8.	IMSSSK	1	57.	KWSSSK	0	105.	KKGVLK	0
9.	LMSSSK	1	58.	KYSSSK	0	106.	KLGVLK	0
10.	MMSSSK	0	59.	KMAAAK	1	107.	KMGVLK	2
11.	NMSSSK	2	60.	KMCCCK	1	108.	KNGVLK	0
12.	PMSSSK	1	61.	KMDDDK	0	109.	KPGVLK	0
13.	QMSSSK	1	62.	KMEEEK	0	110.	KQGVLK	0
14.	RMSSSK	2	63.	KMFFFK	0	111.	KRGVLK	0
15.	SMSSSK	1	64.	KMGGGK	1	112.	KSGVLK	0
16.	TMSSSK	1	65.	KMHHHK	0	113.	KTGVLK	0
17.	VMSSSK	1	66.	KMIIIK	0	114.	KVGVLK	0
18.	WMSSSK	0	67.	KMKKKK	0	115.	KWGVLK	0
19.	YMSSSK	0	68.	KMLLLK	0	116.	KYGVLK	0
20.	AMSSSG	1	69.	KMMMMK	0	117.	KMAVLK	1
21.	CMSSSG	1	70.	KMNNNK	0	118.	KMCVLK	0
22.	DMSSSG	0	71.	KMPPPK	0	119.	KMDVLK	0
23.	EMSSSG	0	72.	KMQQQK	0	120.	KMEVLK	0
24.	FMSSSG	0	73.	KMRRRK	0	121.	KMFVLK	0
25.	GMSSSG	1	74.	KMTTTK	3B	107.	KMGVLK	2
26.	HMSSSG	1	75.	KMVVVK	0	122.	KMHVLK	0
27.	IMSSSG	1	76.	KMWWWK	0	123.	KMIVLK	0
28.	KMSSSG	4	77.	KMYYYK	0	124.	KMKVLK	0
29.	LMSSSG	1	78.	SASSSK	1	125.	KMLVLK	0
30.	MMSSSG	0	79.	SCSSSK	1	126.	KMMVLK	0
31.	NMSSSG	1	80.	SDSSSK	0	127.	KMNVLK	0
32.	PMSSSG	1	81.	SESSSK	0	128.	KMPVLK	0
33.	QMSSSG	1	82.	SFSSSK	0	129.	KMQVLK	0
34.	RMSSSG	2	83.	SGSSSK	0	130.	KMRVLK	1
35.	SMSSSG	1	84.	SHSSSK	0	131.	KMSVLK	2
36.	TMSSSG	1	85.	SISSSK	0	132.	KMTVLK	0
37.	VMSSSG	1	86.	SKSSSK	0	133.	KMVVLK	0
38.	WMSSSG	0	87.	SLSSSK	0	134.	KMWVLK	0
39.	YMSSSG	0	15.	SMSSSK	1	135.	KMYVLK	0
40.	KASSSK	3A	88.	SNSSSK	0	136.	KAGCAK	1
41.	KCSSSK	3B	89.	SPSSSK	0	137.	KCGCAK	0
42.	KDSSSK	0	90.	SQSSSK	1	138.	KDGCAK	0
43.	KESSSK	2	91.	SRSSSK	0	139.	KEGCAK	0
44.	KFSSSK	1	92.	SSSSSK	0	140.	KFGCAK	0
45.	KGSSSK	2	93.	STSSSK	0	141.	KGGCAK	0
46.	KHSSSK	2	94.	SVSSSK	0	142.	KHGCAK	0
47.	KISSSK	1	95.	SWSSSK	0	143.	KIGCAK	0
48.	KKSSSK	1	96.	SYSSSK	0	144.	KKGCAK	0
49.	KLSSSK	2	97.	KAGVLK	0	145.	KLGCAK	0

(continued)

Table 7. **Continued.**

146.	KMGCAK	3A	183.	KMGCLK	3B	219.	KGGGGG	0
147.	KNGCAK	0	184.	KMGCMK	3B	220.	AAAAAK	0
148.	KPGCAK	0	185.	KMGCNK	3B	221.	AAAAAA	0
149.	KQGCAK	2	186.	KMGCPK	0	222.	AMAAAK	0
150.	KRGCAK	0	187.	KMGCQK	3B	223.	AMAAAA	0
151.	KSGCAK	1	188.	KMGCRK	2	42.	KDSSSK	0
152.	KTGCAK	0	189.	KMGCSK	4	224.	KMDSSK	1
153.	KVGCAK	0	190.	KMGCTK	4	225.	KMSDSK	3A
154.	KWGCAK	0	191.	KMGCVK	3B	226.	KMSSDK	4
155.	KYGCAK	0	192.	KMGCWK	3B	43.	KESSSK	2
156.	KMACAK	3A	193.	KMGCYK	3B	227.	KMESSK	1
157.	KMCCAK	1	194.	KMGAAK	2	228.	KMSESK	0
158.	KMDCAK	0	146.	KMGCAK	3A	229.	KMSSEK	3B
159.	KMECAK	0	195.	KMGDAK	1	230.	KMSGSK	4
160.	KMFCAK	0	196.	KMGEAK	0	231.	KMSKSK	4
146.	KMGCAK	3A	197.	KMGFAK	0	64.	KMGGGK	1
161.	KMHCAK	0	198.	KMGGAK	1	232.	KMGGGG	0
162.	KMICAK	0	199.	KMGHAK	0	233.	KMSSGK	4
163.	KMKCAK	0	200.	KMGIAK	2	234.	KMSSGG	4
164.	KMLCAK	0	201.	KMGKAK	1	235.	KMSGGK	1
165.	KMMCAK	0	202.	KMGLAK	0	236.	KMSGGG	1
166.	KMNCAK	0	203.	KMGMAK	0	237.	KMGGSK	4
167.	KMPCAK	0	204.	KMGNAK	3A	238.	KMGGSG	1
168.	KMQCAK	1	205.	KMGPAK	0	239.	KMGSGK	4
169.	KMRCAK	1	206.	KMGQAK	0	240.	KMGSGG	4
170.	KMSCAK	4	207.	KMGRAK	1	59.	KMAAAK	1
171.	KMTCAK	1	208.	KMGSAK	3A	241.	KMAAAA	1
172.	KMVCAK	0	209.	KMGTAK	3A	242.	KMSSAK	4
173.	KMWCAK	0	210.	KMGVAK	2	243.	KMSAAK	3B
174.	KMYCAK	0	211.	KMGWAK	0	244.	KMAASK	4
146.	KMGCAK	3A	212.	KMGYAK	1	245.	KMASAK	3B
175.	KMGCCK	3B	92.	SSSSSK	0	246.	KSMSSK	0
176.	KMGCDK	2	213.	SSSSSS	0	247.	KSSMSK	0
177.	KMGCEK	2	214.	SSSSSG	0	248.	KSSSMK	2
178.	KMGCFK	3B	88.	SMSSSK	1	249.	KMGACK	3A
179.	KMGCGK	3B	215.	SMSSSG	1	250.	KMGVCK	3B
180.	KMGCHK	3B	216.	GGGGGK	0	251.	KMGVPK	1
181.	KMGCIK	3B	217.	GGGGGG	0			
182.	KMGCKK	1	218.	KGGGGK	0			

Left: Sequences of mutants, Right: degrees of activities by amber suppression (in order of increasing activity: 0, 1, 2, 3B, 3A, 4).

three different chloramphenicol-free culture media, respectively. Then, such the culture media mixed with the colonies are spotted on the culture plates containing chloramphenicol. LB culture plates of five different chloramphenicol concentrations as 450, 300, 200, 100, 50 and 25 μg/ml were prepared, and the spotting tests were performed twice for each mutant for the verification.

Typically, colonies with wild type clones of MjYRS would survive on the plate with 300 μg/ml or 450 μg/ml chloramphenicol. However, absolute values of chloramphenicol concentrations of the plate from which the colonies survived is vary by each examination. It is difficult to evaluate catalytic abilities of mutants based on such the absolute value. Therefore, we decided to evaluate catalytic abilities of mutants in relative manner with each others. Though evaluations in this and following sections, we defined five degrees as 4–0 for the catalytic ability, and the degree of the wild-type sequence KMSSS is defined as 4.

In Vitro Analyses of Aminoacylation—Mutants and wild-type of MjYRS were cloned within the *NdeI–XhoI* sites of pET41a(+) vector (Novagen) to generate the plasmid pET-TYR-His. MjYRS mutants and wild-type MjYRS were overproduced in the BL21(DE3) cells (Novagen). The proteins were purified by chromatography on Ni–nitrilotriacetic acid (NTA) agarose (Qiagen). tRNA for the Amber suppression was prepared by runoff transcription using T7 RNA polymerase (*30*). In short, RNase P components of C5 protein and M1 RNA were expressed and purified. Then, precursors of tRNAs (Pre-tRNAs) with 27 nt hummer head region were transcribed by T7 RNA polymerase. Pre-tRNAs were processed to cut the hummer head region with RNase P and generate target tRNAs.

tRNA was renatured at 65°C for 5 min in 10 mM HEPES–KOH (pH 7.5) and 15 mM $MgCl_2$ before aminoacylation, followed by slow cooling to room temperature for 2 h. For acidic page, the aminoacylation was performed at 37°C for 30 min in 50 μl of buffer A (100 mM HEPES–KOH, pH 7.5, 15 mM $MgCl_2$, 1mM DTT, 0.05 mg/ml BSA) containing tRNA for the Amber suppression (5 μM), 10 mM ATP, L-tyrosine (0.1mM), and TyrRS mutant (30 or 150 μg/ml). Analysis of the aminoacylated tRNAs was carried out by acidic PAGE (*32*, *33*) using a denaturing gel [7% acrylamide, 40% Urea, 0.1 M NaOAc pH 5.0, 0.67 mg/ml Ammonium persulfate (Nacalai Tesque), 0.033% Tetramethylethylenediamine (Nacalai Tesque)]. The tRNA in the gel was stained with Methylene blue (Chroma).

To obtain kinetic parameters, tyrosylation of tRNAs with radioisotope was performed in 100 mM HEPES–KOH (pH 7.5), 15 mM MgCl2, 1 mM DTT, 0.05 mg/ml BSA, 10 mM ATP, 11 μM L-[U-^{14}C]Tyrosine (GE Healthcare) with various tRNA and TyrRS concentrations for the Amber suppression at 37°C for 1–30 min. After incubation, aliquots were removed, spotted on Whatman 3 MM paper, and precipitated by trichloroacetic acid. Incorporation of radioactive tyrosine to tRNAs was measured by liquid scintillation counter (*31*) (Perkin Elmer) at Radioisotope center, the University of Tokyo. Lineweaver–Burk plots were used for calculating the kinetic constants, and averages of constants were calculated from at least three independent experiments.

RESULTS

Evaluations of Selected Mutants from the Randomized Mutant Library—Colony selection from randomized mutant library was performed by the Amber suppression method on the culture plate containing 300 μg/ml chloramphenicol. All the colonies selected from the plate were sequenced, and statistics of expressions were obtained concerning each residual number (Tables 3–6).

At first, residuals of the number 202, 203, 204, 208, 209 and 210 were randomly mutated, and colonies were selected by the Amber suppression screening (Tables 3 and 4). From the statistics of appeared expressions, there seems to be poor correlations between the characteristics of the amino acids and the appearance frequencies at the residual number 202, 203, 209 and 210. As results, the fact was confirmed that the KMSKS motif consists of the five successive residues of KMSSS as revealed in the previous works on motif search (*1–6*). At the same time, the fact as those mutations allowing various alternatives suggests that mutations around the KMSSS loop would not affects main frame structure of the TyrRS. Therefore, it became possible to investigate variations of the motif focusing on the five residue of the KMSSS loop and to discuss the catalytic conditions according to characteristics of the five residues themselves. Residuals of the number 204–208 were randomly mutated, and colonies were selected by the Amber suppression screening (Tables 5 and 6). About each mutant clones from the selected colony, activation degree was examined individually (Tables 5 and 6). The examinations were performed by the spotting method for the Amber suppression. This verification process was employed in order to avoid selections of the colonies with false activities. The most of the degrees can be classified into two groups, and seventy-one out of seventy-six mutants represented the degree of 3 or 4. Therefore, we acquired statistics of the mutation about those 71 mutants.

From the statistics, some important rules appeared as follows.

1. Lys at residual number 204 was not replaced by other amino acid residues.
2. Residual number 205 has a partial restriction for the mutations to keep activities.
3. 90.8% of expressions at residual number 206, 207 and 208 were occupied by Ser (43.1%), Gly (14.9%), Ala (14.4%), Thr (10.3%) and Cys (8.0%).
4. Asp and Glu did not appear at any positions.
5. From the mutation of 'KMXXX', most of mutants include at least one Ser or Thr at residual number 206, 207 and 208. However mutants including neither Ser nor Thr were found as KMGCA, KMGAC and KMGVC.

Above statistics suggest hypotheses as follows:

1. Positively charged side chains are important to bind ATP which is negative-charged because of phosphate groups, negatively charged side chains are not allowed because they should repel the ATP with negatively charged phosphate groups.
2. Ser, Gly, Ala and Cys are preferable in order to keep the loop flexible due to their small side chains.
3. Although Ser is an important amino acid for activation of the loop, Cys could be a substitution for Ser.

Evaluation of Specifically Designed Mutants—In order to verify above hypotheses, we specifically designed mutants to rebuild the library, and examined their catalytic abilities individually (Table 7). Activity of each mutant was examined by the spotting method for the Amber suppression, and its activity was classified into five degrees of 4–0. Among the five degrees, degree 4 represents the most high activity as same as the wild-type, and degree 0 represents no activity for the Amber suppression. Mutants representing higher activity than the wild-type have not been acquired in the experiments.

Mutants 1–19 were designed to verify importance of Lys204. All of the mutants represented lower activity than the wild-type MjYRS. However, mutants substituted from Lys204 to Arg and Asn represented relatively high degree of activities among the mutants 1–19. Since Arg has a similar side chain as Lys, the result suggests the importance of the common property of the side chain of Lys and Arg. On the other hand, mutants 20–39 were designed to investigate the contribution of Lys209 to the loop activity. The results were almost same as the results from mutant 1–19. The results suggest that Lys209 has almost no contribution to the loop activity. The results of colony selection from randomized library support the suggestion as poor contribution of Lys209 (Table 3). Therefore, it is possible to discuss the loop activity without concern about the contribution of Lys209.

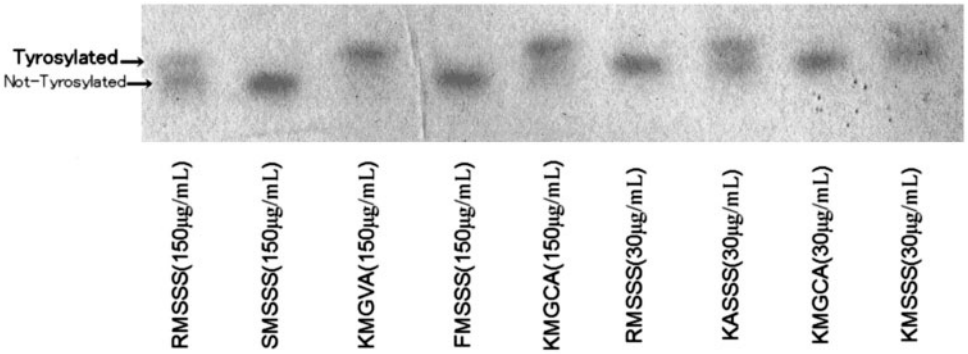

Fig. 1. **Results of Acidic PAGE: wild type and mutant proteins diluted to 30 and 150 μg/ml.**

Mutants 40–58 were designed to investigate contribution of Met205 to the loop activity. Mutations from Met205 to Ala and Gln have maintained the activities to the degree of 4 as same as the degree of the wild type, and the mutation to Cys, maintained the degree of 3. Clu, Gly, His, Leu and Thr have lowered their catalytic abilities to the degree of 2. Among those amino acids, there seems to be poor restrictions about mutations of Met205.

Mutants 59–77 were designed to search alternatives for Ser206-Ser207-Ser208. Only the mutation to Thr206-Thr207-Thr208 maintained the activity to the degree of 3, where Thr has a hydroxyl group in the relatively small side chain like Ser. Besides them, mutants 97–212 were designed to further investigate the variations from mutants KMGVL, KMGAC and KMGCA which

In Vitro Analyses of Aminoacylation—In the strict manner, the activity of aminoacyl-tRNA synthetase should be measured directly by the degree of aminoacylation itself. However, we employed more simple method such as the Amber suppression method than direct measurement of aminoacylation which requires purification of more than two hundred mutant proteins. In order to verify the correlation between the degrees of aminoacylation and the amber suppression, we selected representative mutants from the library, and purified the proteins.

We then performed aminoacylation of the tRNA (MjR1) by those representative mutants of MjYRS, and visualize the results by acidic PAGE (Fig. 1). Bands of aminoacylated tRNA by the mutants having activity of degree 4 and 3 were recognized slightly above the bands of not aminoacylated tRNA by the mutants having activity of degree 0. Since degrees of aminoacylation and amber suppression seem to have good correlation, discussions based on the amber suppression can be regarded as equivalent to discussions based on aminoacylation itself.

In the concentration of 150 μg/ml for the mutant proteins, bands corresponding to aminoacylated tRNA appeared for the mutants of the catalytic degree 4, but the bands did not appear for the mutants of the degree 3 and 2 (Fig. 1). In the concentration of 30 μg/ml for the mutant proteins, the bands appeared for the mutants of the degree 4 and 3, but the bands did not appear for the mutants of the degree 1 and 0 (Fig. 1). Although the band for the mutant of the catalytic degree 2 appeared in the concentration of 30 μg/ml, the ability of aminoacylation seemed to be poorer than the mutants of the degree 3. The mutants of degree 3A sometimes represent the same aminoacylation ability in the following Acidic PAGE assay. Although the mutants of degree 3B have close catalytic activities to the wild-type, it hardly represents the same or more activities than the wild-type.

Table 8. **Kinetic parameters for tyrosylation by wild-type and mutant TyrRS.**

	K_{cat} (s^{-1})	K_m (μM)	K_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)
KMSSS (wild-type)	0.53	4.3	0.12
KMGCA	0.025	1.5	0.017
RMSSS	0.010	4.3	0.0023
AMSSS	0.012	4.4	0.0027
FMSSS	ND	ND	ND

ND, not determined due to low activity.

We also obtained kinetic parameters for wild-type and mutant TyrRS (Table 8). While K_m values were similar among the wild-type and mutants and only KMGCA mutant show slightly better K_m, K_{cat}/K_m values corresponded approximately to degrees of resistance to chloramphenicol obtained from amber suppression experiments. From those results, the degree of the amber suppression evaluated by the degree of aminoacylation and the degree of resistance to chloramphenicol seem to have good correlation with each others.

DISCUSSION

As described in the preceding section, the fact as those mutations allowing various alternatives suggests that mutations around the KMSSS loop would not affects main frame structure of the TyrRS. Therefore, it became possible to investigate variations of the motif focusing on the five residue of the KMSSS loop and to discuss the catalytic conditions according to characteristics of the five residues themselves. On the other hand, it is possible that mutations affect the loop structure itself to prevent proper catalytic activity. However, such the

situation can be considered to some extent by comparing with data on other mutants.

Alternatives for K204—In the results of colony selection from our of random PCR library, Glu and Asp did not appear in the substitution of the five residues of the KMSSS loop. In order to verify such the suggestions from randomized library, mutants 1–19 were designed by mutating Lys204 to the other residues (Table 7). Mutations of Lys204 to Asn and Arg maintained catalytic abilities the mutants at the degree of 2, which is more than the degree of the other mutations. From the results of those two different experiments, it is assumed that positive charge of the first Lys is important for ATP interaction. The other interesting point is that eleven samples out of the mutants 1-19 keep the activation degree of 1. Therefore, the loop is able to maintain catalytic ability to some extent even when the loop lacks the first Lys. However, it appeared that Lys204 should be strictly restricted by comparing the degree of mutants 54 and 86.

Besides them, mutants 224–229 were individually designed to examine the influences from Glu and Asp to the loop. The results indicate that catalytic abilities were lowered if the distance of the negatively charged residues as Glu and Asp from Lys204 becomes closer. Those facts suggest that existence of negatively charged residues as Glu and Asp in the loop degrades its catalytic ability.

On the other hand, mutants 20–39 were designed to investigate the contribution of Lys209 to the loop activity. From those results, it is suggested that Lys209 has almost no contribution to the loop activity. The results of colony selection from randomized library support the suggestion as poor contribution of Lys209 (Tables 3 and 4). Selected colonies in the experiments on 'KMSSXxx' (Tables 3 and 4) suggested that a variety of mutations were allowed for Lys209. Therefore, it is possible to discuss the loop activity without concern about the contribution of Lys209.

Alternatives for Met205—Mutants 40–58 were designed to investigate contribution of Met205 to the loop activity. Mutations to Ala and Gln have kept the activities to degree 3 same as the wild type, and mutations to Cys, Glu, Gly, His, Leu and Thr have kept activates to degree 2. Among those amino acids, there seems to be poor restriction on mutations. On the other hand, mutants 136–155 were also designed to investigate contribution of Met205 to the loop activity. Ser206-Ser207-Ser208 were mutated to Gly206-Cys207-Ala208 about mutants 136–155 being derived from the mutant 146(KMGCA) which was selected from randomize library (Table 5). Mutants 136–155 were designed in order to search the sequences that have similarities to the wild-type sequence as poor as possible, while keeping the activity to the same degree as the wild-type. As results, only Gln kept the activity to degree 3. Thus, mutations of Met205 in mutants 136–155 seem to have more severe restrictions than mutants 40–58.

Besides them, mutants 246–247 were designed to investigate restrictions for the position of Met in the loop. From the results of mutants 246 (KSMSS) and 247 (KSSMS), there seems to exist a restriction on the position of Met. Although mutant 248 (KSSSM) represented activation degree 3, it seems reasonable that mutant 248 (KSSSM) is designed by mutating the least influential part of mutant 54 (KSSSS) and they represent similar activation degree.

This result suggests that mutation of Met has restrictions to some extent, but Gln is highly alternative to Met. Importance or restrictions of Met205 have not been discussed in the related works. Although the reason for restriction cannot be revealed in this paper, the fact of alternation to Gln would be an important suggestion to the theoretical researches. Furthermore, it was remarkable to obtain the sequence 'KQGCA' which is the poorest similarity to the wild-type sequence 'KMSSS'. Such the sequence has never been obtained by homology search of the motif from protein databases.

Alternatives for Ser206-Ser-207-Ser208—In the previous works (*7–14*), importance of Ser in the loop for ATP binding has been discussed through the structural analyses and theoretical analyses. In order to verify the discussions in the previous works, we specifically designed mutants 59–77 and 97–212.

From the results in Tables 5 and 7, most of the mutants having at least one Ser at the position of 206, 207 and 208 represented catalytic ability of the degree 4 or 3. In addition, there appeared no mutant without Ser or Thr among the mutants having catalytic degree of 4. Therefore, the suggestion from the previous works that the Ser and Thr are important for the motif was verified by our experiments. However, there does not seem to have strict restriction in the position of Ser and Thr.

However, it is remarkable that there appeared the mutants as KMGCA, KMGNA, KMACA and KMGAC among the mutants having catalytic degree of 3A. Although those four mutants did not have any Ser or Thr in the loop sequence, they represent quite similar activities to the wild-type loop. Common characteristics of those four sequences are that they contain residues as Cys or Asn which have polarized side chains as S-H or C = O groups. Besides them, no mutant without residues with polarized side chains has maintained its catalytic degree of 4, 3A or 3B. From those results, residues 206–208 seem to accept flexible mutation, but at least one residue with polarized side chain is required among residues 206–208. The position of such the residue with polarized side chain does not seem to be restricted. In the previous work (*14*), importance of hydrogen bonds is discussed about Ser206 and Ser208, and experimental results in this article support this discussion. However, experimental results revealed that at least one Ser can maintain the catalytic activity of the loop, and its location is flexible. It was also revealed that Cys and Asn can substitute Ser to a certain degree.

From the results of the mutants 146 and 175–193, it was suggested that the fifth residue of the KMSSS loop, that is Ser208, is the least important among those five residues. Results from the mutants 42, 43, 224–226 and 227–229 support this suggestion where the mutant 226 (KMSSD) has the catalytic degree 4, and the mutant 229 (KMSSE) maintained the catalytic degree of 3B.

The results in Table 5 also support this suggestion where various alternatives for Ser208 appeared from the random mutation as KMSSXxx.

As a summary, at least one polarized amino acid is required among residues 206–208, and its location is flexible. The other residues of 206–208 prefer amino acids as Gly and Ala to keep the loop flexible and not to disturb the contribution of Lys204 to the catalysis. Since the residual position 208 is far from Lys204, it accepts various amino acids as Phe, His, Trp and so on. It is reasonable that residues of the loop do not accept Pro which notably disturb the loop to keep flexible.

CONCLUSION

In general, pattern classification requires a lot of learning data to be classified into every class. In other words, designing pattern recognition algorithm requires learning data of both to be detected and not to be detected by the algorithm. Today's bioinformatics approaches in protein science generally employ statistical analyses of the databases based on sequences and structures of natural proteins. Those approaches have been proved to be quite successful in finding motifs and other conserved sequences which are important for the catalytic activation of proteins. However, such the conserved sequences have been influenced from the initial state, and there may exist other sequences which represent similar activity as such the conserved motifs. Besides them, it is important to know the sequences which lose the activity of the motif, and to investigate the boundaries between the sequences maintained the activities and the sequences lose the activities.

In this article, we investigated the various mutants of the KMSSS loop of tyrosyl-tRNA synthetase of *M. jannaschii*, and obtained other sequences with similar activities as the wild-type sequences. Some sequences obtained have quite different sequences from wild-type sequences. From those data, we could found some suggestions about boundary between active and inactive sequences. In order to reveal concrete law for such the boundary, theoretical or computational works are necessary. The data obtained from this article will be useful for those further works.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

None declared.

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